INTRODUCTORY NOTES ON
CHROMATOGRAPHY

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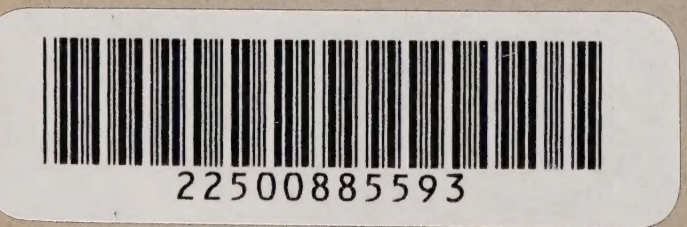

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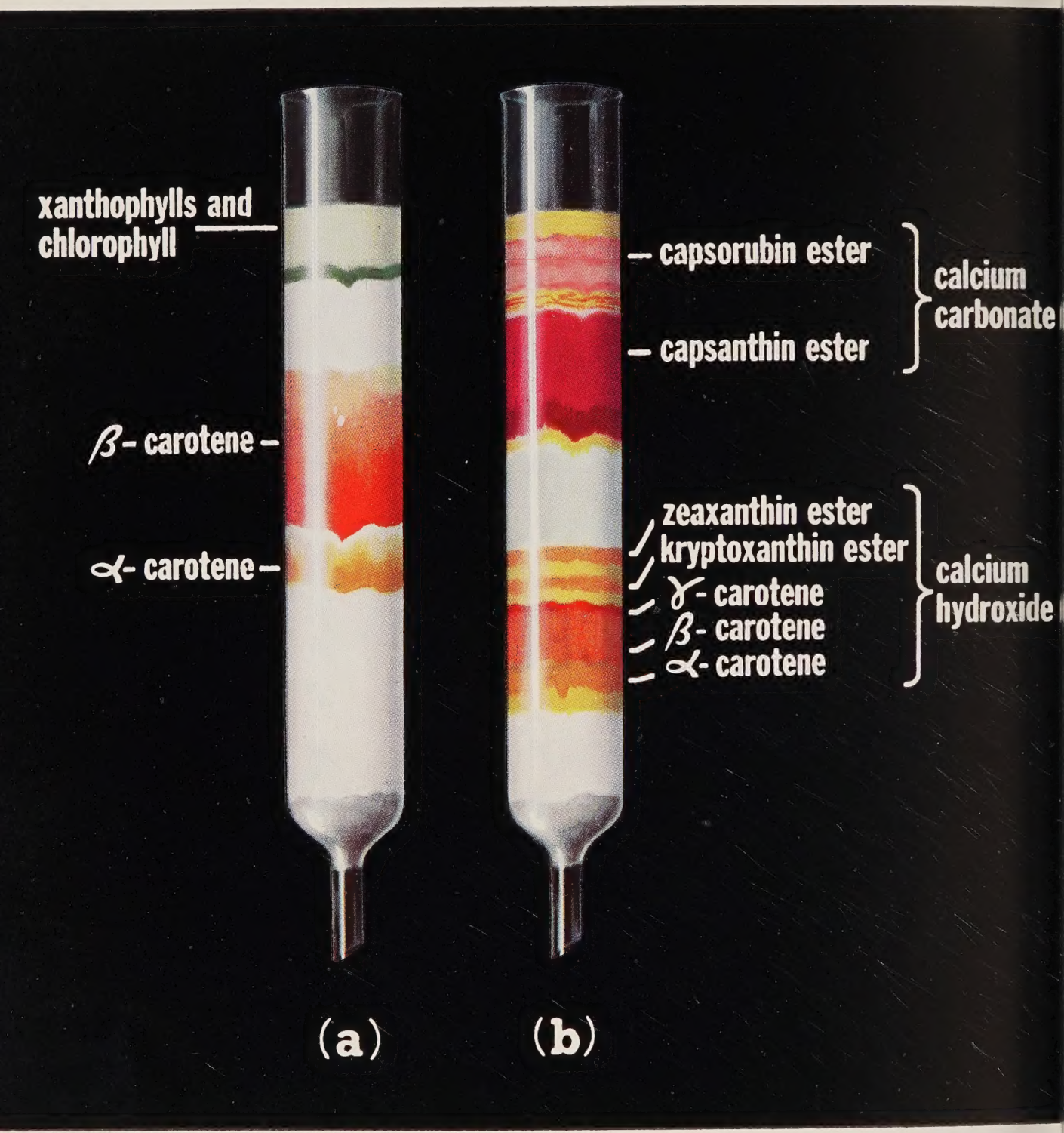
SEPARATION OF CAROTENES AND PAPRIKA PIGMENTS



(*a*) The separation of carotenes by passing a light petroleum extract of green leaf material through magnesia.

(*b*) Separation of the pigments of paprika on a composite column of calcium carbonate and calcium hydroxide, using a light petroleum extract of the powdered fruit of *Capsicum anuum* (after L. Cholnoky, *Kisérletügyi Közlemények*, 1937, **40**, 173).

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FOREWORD

As its title indicates, this booklet is merely intended to provide a convenient means of introducing the subject of Chromatography to those who have not hitherto given it particular attention. While the treatment has necessarily been cursory it will be noticed that some of the applications have been described with more detail than one might expect to find in a monograph of this nature. Such extended accounts have been included wherever it appeared that they would help to illustrate methods suitable for use in analagous cases. Thus, particular attention has been accorded to the account of the part played by Chromatography in early work on the isolation of vitamins A and D and, again, the recent developments on Partition Chromatography using paper supports have been described at sufficient length to give an indication of their importance.

At this stage opportunity may be taken to emphasize a statement to be found in the text that the practice of Chromatography does not constitute a principle of analysis in the sense applied to gravimetric, volumetric or colorimetric methods because it is never possible to conduct an analytical determination chromatographically to the exclusion of other procedures. It would appear, therefore, that the term " chromatographic analysis " is not a happy one and might well be avoided.

Readers desirous of studying the subject of Chromatography in detail are recommended to consult the following works :—

Principles and Practice of Chromatography by L. Zechmeister and L. Cholnoky : translated from the German by A. L. Bacharach and F. A. Robinson (*Chapman and Hall, Ltd., London, 1941*).

Chromatographic Analysis, by A. H. Cook (*The Institute of Chemistry, London, 1941*).

Chromatographic Adsorption Analysis, by Harold H. Strain (*Interscience Publishers, Inc., New York, 1941 : revised reprint 1945*).

An Introduction to Chromatography, by Trevor I. Williams (*Blackie and Son, Ltd., London, 1946*).

Symposium on Chromatography. (*The Society of Public Analysts and W. Heffer and Sons, Ltd., Cambridge, 1947*).

Much of the information in this monograph is to be found in one or other of the above publications and indebtedness for help derived from their pages is freely acknowledged. Finally, we desire to express our thanks to Dr. K. A. Williams for kindly checking the manuscript and providing many valuable suggestions.

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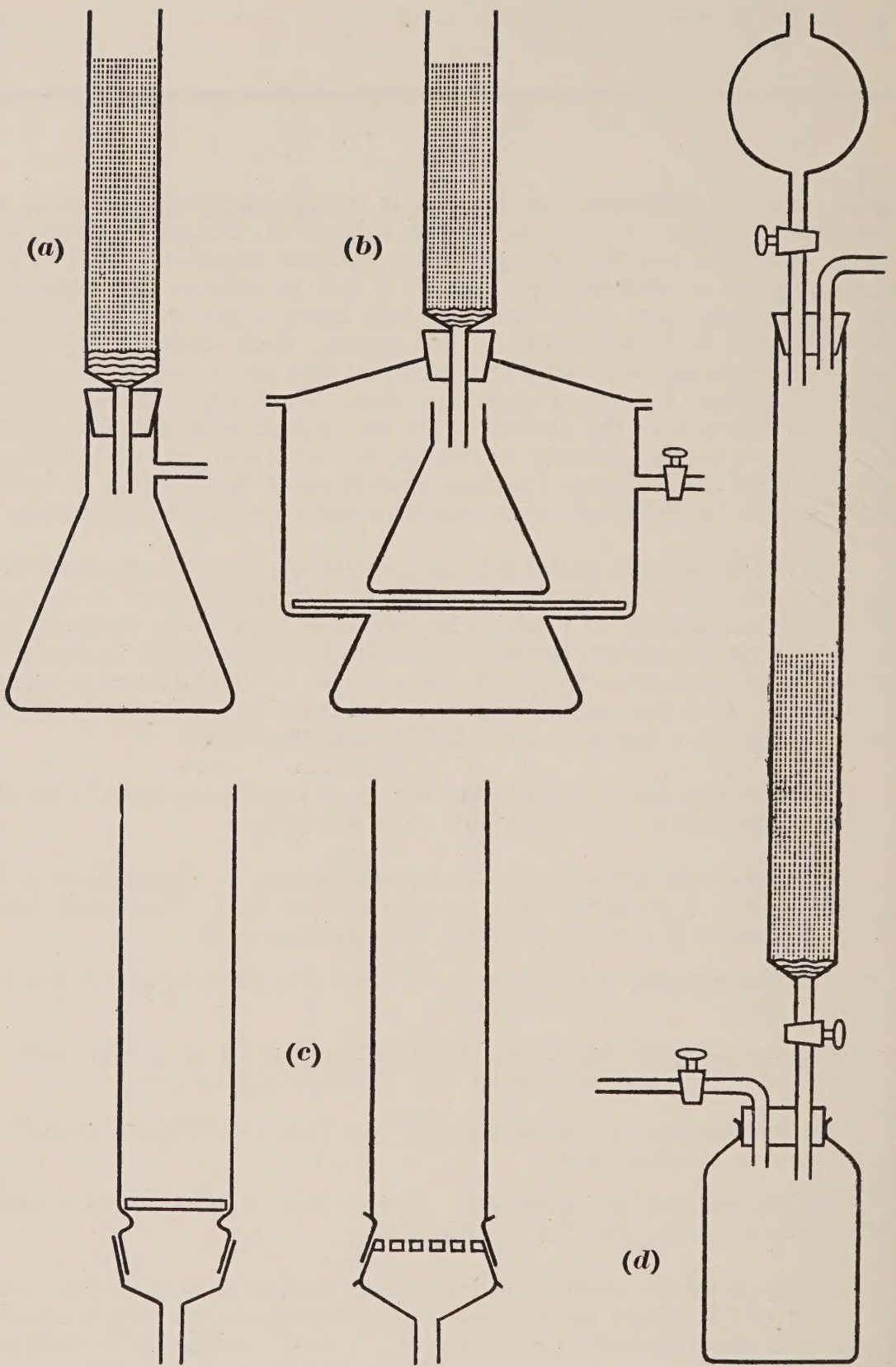
April, 1948.



(*a*) Simplest form of adsorption apparatus for use with suction
(*b*) Arrangement for collecting several fractions of percolate
(*c*) Types of adsorption tube fitted with movable perforated discs
(*d*) Apparatus for work in an inert atmosphere

INTRODUCTORY NOTES ON

CHROMATOGRAPHY

Part I — *General Principles*

THE chromatographic method was originated in the year 1906 by the Russian botanist M. Tswett who employed it for the purpose of separating the pigments of plants into their constituents.[1] In general, the procedure involves the following operations:—

1. Preparation in a suitable solvent of a solution of the mixture under examination.

2. Percolation of the solution through a column of an adsorbent powder, such as activated alumina.

3. Separation of the material adsorbed on the column into horizontally disposed bands of the individual chemical compounds: this step, which is referred to as "the development of the chromatogram", is effected by washing through with a solvent, which may or may not be the same as that used to prepare the solution of the sample.

4. Removal of the column from the percolator and separation of the bands by appropriate mechanical division of the adsorbent.

5. Agitation of the separated zones of adsorbent with suitable polar solvents, which when used in this connection are known as eluants.

6. Clarification of the eluants by filtration or centrifugation and recovery of the resolved compounds by evaporation or other suitable procedure.

Additional to the above technique, the method is often used for removing by adsorption undesired substances present in solutions from material which it is required to isolate and in these cases it is the first percolate which is of most interest, this being evaporated to yield a residue characterised by its capacity to pass through the column without being adsorbed. This offers a convenient principle for the separation of hydrocarbons from vegetable oils. In certain cases the chromatographic principle offers the only known means of resolving mixtures into their components. For example, in 1931 Kuhn and Lederer[2] separated the

reddish-yellow plant pigment carotene into two isomeric hydrocarbons, α and β carotene, in sufficient quantity to allow of ultimate analyses being conducted by the Pregl micro-combustion procedure. After the publication of this work, chromatography, which had been largely neglected since the appearance of Tswett's last paper in 1912, at once became established as a manipulative principle to be widely exploited. It is not often possible to attain quantitative recoveries of individual constituents of mixtures by the chromatographic method but under favourable conditions losses may not amount to more than 2 to 5 per cent. although in other cases the yield of a particular component may be no more than a few per cent. of that present in the amount of mixture passed through the column.

Certain general rules have been deduced relating the adsorbability of organic compounds with the structure of their molecules and it has been observed that the presence of polar groups favours adsorption. This fact is illustrated by the following series in which the most strongly adsorbed types are listed at the top and the least adsorbed compounds at the bottom:—

Acids and bases
Hydroxyl, amino and thio compounds
Aldehydes, ketones and esters
Halogen compounds
Unsaturated hydrocarbons
Saturated hydrocarbons

It should be observed, however, that it is not possible to separate all types of organic compounds into their structural categories by the use of chromatographic methods because the adsorbability varies with the size of the molecule, large molecules usually being more strongly adsorbed than small ones of the same class. Again, variations in adsorbability occur with stereoisomeric change and with alterations in the polarity of substituent groups depending upon their positions in the molecule. Homologous series of unsaturated organic compounds reveal an increase in adsorbability with the number of double bonds. Amongst isomeric compounds such as the polycyclic aromatic hydrocarbons, however, other factors such as the shape and polarity of the molecule exercise an influence which, in some cases, amounts to more than that due to the double bonds alone. Thus, 1 : 2-benzpyrene, with five six-membered rings and ten double bonds is less easily adsorbed than is naphthacene, having four six-membered rings and nine double bonds, a circumstance probably due to the fact that, if we consider both compounds as derived from anthracene, in the first the attached ring system occupies one of the meso positions of the anthracene molecule while the naphthacene molecule, being linear, has both these positions free.

Apparatus

The simplest and most widely used apparatus consists of a glass tube, say, 35 cm. long by 1 cm. to 2 cm. internal bore, constricted at one end and mounted by means of a rubber bung over an ordinary suction

filtering flask. A plug of cotton wool is pushed down to the constricted end and the tube then charged for about two-thirds of its length with the adsorbent powder. For the examination of fluorescent materials it is sometimes advantageous to use quartz tubes because of their high transmission of ultraviolet light. In industrial operations columns of opaque materials such as stainless steel and porcelain are frequently used. Numerous modifications and additions to the essential apparatus have been recommended by various investigators for use in particular researches, but usually these follow on conventional lines and do not call for special explanation. However, a refinement in which the adsorption tube, instead of being constricted at the lower end, is made to fit a ground glass joint and the cotton wool plug is supported upon a movable perforated disc, as shown on page 4, is worth mentioning since its use facilitates the removal of columns after development of the chromatograms.

Procedure

While cotton wool will generally serve as a support for the adsorbent, cases arise in which this material is attacked by the percolates and it is then necessary to employ an alternative such as glass wool, plates of fritted glass or porcelain discs. In any event, it is important that the support should extend above the constricted portion of the glass tube in order to ensure even distribution of suction at the base of the column, this being essential for uniform percolation of the solution and formation of definite bands.

Considerable care must be exercised in charging tubes in order to ensure homogeneity of the adsorbent column. While coarse powders may simply be poured in as the sides of the tube are continuously tapped, moderately fine powders should be added in small successive portions and each firmly tamped into place with a plunger. Finely divided adsorbents tend to form columns through which liquids percolate only slowly and when this is liable to occur the powder is usually mixed with larger particles of the same adsorbent or with fibrous inert fillers. When the adsorbent is resilient it is better to press, rather than tamp, it into the tube in small layers at a time. In order to obtain satisfactory chromatograms it is often necessary to introduce the adsorbent in the form of a slurry made with the solvent to be used to dissolve the sample under examination. In this connection A. L. Levy recommends placing the adsorbent and solvent in a separating funnel fitted with an efficient link stirrer which is rotated, whence the resulting slurry is allowed to flow through the tap into a clean, dry tube.[3] The adsorbent settles fairly rapidly and the process may be aided by tapping the tube or by applying gentle suction at the base. When a tube is charged with adsorbent previously mixed with a solvent the column must not be sucked dry as otherwise it will shrink from the sides of the tube thus rendering the assembly useless. Sometimes it is convenient to minimise the disturbance of the upper portion of the column occasioned by the addition of solvent or solution by placing a wad of cotton wool, a perforated disc, or a sheet of filter paper on top of the packed adsorbent, but this practice may result in the formation of uneven bands of the adsorbed substances.

If the tube is charged with dry adsorbent the latter must be completely soaked with the solvent used for dissolving the sample before the test solution is added. From this stage until the chromatogram is developed it is most important to maintain a layer of solvent, or eluant as the case may be, above the adsorbent so that air is not drawn into the upper portions of the column. For the resolution of mixtures, it is usually a good plan to begin with fairly strong solutions, whence the solute tends to be adsorbed in a shallow band in the upper portion of the column thus leaving adequate room for the development of the chromatogram. It is important to avoid adding too much material as otherwise no space will remain in the column for effecting separations and in preliminary experiments with unknown mixtures it is better to adsorb too little than too much. So soon as the solution of materials to be resolved has been drawn into the adsorbent, the column is washed with fresh portions of the same solvent as that from which the compounds were adsorbed. This treatment generally causes the adsorbed constituents of the original sample to move down the column at different rates but it may happen that a more polar solvent is needed to develop the chromatogram. For example, substances adsorbed from petroleum ether may be washed subsequently with mixtures of this solvent and a small proportion of alcohol. In any one case it is generally necessary to make many trials before satisfactory separations are effected since, even if the right adsorbent has been selected, the choice of solvents is usually a highly critical matter.

Assuming that a satisfactory separation has been achieved and that it is desired to isolate one or more constituents of the original sample, the column is freed as far as possible from solvent by suction and then extruded from the tube. This operation is generally easy to carry out because when the bulk of the solvent is drawn off the column shrinks and it is readily pushed out by means of a rod. The required zones are then separated and agitated with suitable polar solvents that elute the desired compounds from the adsorbent. The solutions thus obtained are clarified by filtration or centrifugation and the dissolved substances isolated by evaporation of the solvent or other suitable procedure. With mixtures of compounds that exhibit great differences in the degree to which they are adsorbed, the resolved compounds may be isolated by a combination of methods. Thus it may happen that the weakly adsorbed constituents can be washed right through the column and then be recovered from the percolate while the more strongly adsorbed substances are isolated by the procedure already outlined above. Again, when soluble adsorbents are used, these may be dissolved and the liberated compounds recovered from the resulting solution.

Adsorbents

Hundreds of substances have been tried as adsorbents but only a few have proved to be of any practical value. Ideally, an adsorbent should hold relatively large quantities of the materials to be resolved and these should be carried through the column with fresh portions of the solvent in order to permit development of the chromatogram. Further,

the adsorbed substances should be eluted completely by polar solvents, while the adsorbent must not decompose the adsorbed compounds nor any of the solvents used and neither, under the usual conditions, must it dissolve in any of these solvents. Again, the adsorbent should preferably be white and must be of such particle size as to allow of reasonably rapid and uniform percolation. The adsorptive capacity of any given substance often depends upon the method of its preparation and in some cases may vary by several thousandfold according to the preliminary treatment that has been applied. Again, the efficiency of an adsorbent varies with different solvents so it will be realised that it is not possible to arrange the substances used to make chromatographic columns in order of their adsorptive capacity but in the following notes the most generally used adsorbents are first considered.

Alumina. Partially hydrated alumina is one of the best adsorbents and is certainly the most widely used. It may be activated by heating for some time at dull red heat, allowing to cool in air and rubbing through a sieve of 150 mesh. Starting with technical alumina Brockmann and Schodder claim to have prepared five grades distinguishable for their varying adsorptive capacities.[4] Alumina I was prepared by calcination of small portions of the technical product. Product II was obtained by heating technical alumina in a layer 3 cm. deep in a large pot over a burner for from 4 to 6 hours and then allowing to cool for about 30 minutes. Aluminium oxides III, IV and V were prepared from II by exposure to moist air in a cellar until the desired acitivity was obtained. The adsorptive capacity was measured by observing the separation of various dyes in columns 10 cm. long and 1·5 cm. in bore. For each test 20 mg. each of two dyes were dissolved in 10 ml. of pure benzene, the solution diluted to 50 ml. with petroleum ether and 10 ml. passed through the columns followed by 20 ml. of the same solvent free from dye. Alumina I possessed the desired activity when *p*-methoxy-azobenzene (upper) and azobenzene were separated before the latter coloured compound had passed through the column. With alumina II, of lower adsorptive capacity, azobenzene washed completely through under the above test conditions but Sudan yellow (upper) and *p*-methoxy-azobenzene should separate on the column. The correct activity for preparation III was reached when Sudan red could be separated as the upper layer from Sudan yellow, still adsorbed as a lower band. Alumina IV, permitted to remain longer in contact with moist air than III, should fail to hold Sudan yellow but should be capable of separating amino-azobenzene and Sudan red into distinct upper and lower bands respectively. Finally, the weakest adsorbent of the series, product V, exposed to moist air for the longest time, was considered to be of suitable activity provided *p*-hydroxy-azobenzene would form a distinct zone above a band of *p*-amino-azobenzene.

It will be seen from the above that some control over the adsorptive capacity can be exercised and in critical work it may sometimes be a good plan to activate material under known and reproducible conditions starting with alumina which has been deactivated by soaking with water and allowing the wet oxide to dry slowly in the air.

In order to preserve alumina from deactivation by the adsorption of

moisture V. H. Booth [5] recommends mixing with an equal weight of granular anhydrous sodium sulphate (previously heated for 12 hours at 140° C.) and leaving the preparation for 3 days to mature. By the use of this mixture it is claimed that carotene can be separated readily from all other plant pigments.

Magnesia. Material for chromatographic work is made by igniting magnesium hydroxide. It is a fairly powerful adsorbent but special care is necessary in its use owing to its strongly alkaline character. It has been recommended for the resolution of mixtures of xanthophylls.[6]

Quick Lime. This is a fairly powerful adsorbent but different specimens vary considerably according to their method of preparation and degree of purity.

Slaked Lime. When freshly prepared from quick lime by the cautious addition of water this substance is a good adsorbent but has the disadvantage of alkalinity and uncertainty of action.

Calcium Carbonate. When using this mild adsorbent it is important to ensure that it is quite free from moisture and to this end it should always be dried at 150° C. before use.

Barium Carbonate. When freshly dried this compound exhibits slightly more powerful adsorptive capacity than calcium carbonate.

Magnesium Carbonate. The ordinary basic carbonate (heavy variety) is quite frequently used but owing to the extreme fineness of the powder it is often necessary to mix it with a siliceous filtration aid, such as acid-washed kieselguhr, in order to obviate unduly slow percolation.

Talc. This weak adsorbent is said to be useful in the resolution of mixtures of organic acids and also for separating 2 : 4-dinitrophenyl-hydrazones. It is usually necessary to mix it with a filtration aid before packing into columns.

Adsorptive Clays. Certain naturally occurring earths when purified and sifted make useful adsorbents and are, or have been, marketed under proprietary names. Thus Frankonit KL originated from Kitzingen-am-Main, Germany, and Floridin XS, belonging to the same group, is available in this country. The adsorptive clays are all acid and it is often difficult to elute adsorbed substances from them. It has been suggested that this latter feature is connected with the relatively high iron content and that the adsorptive capacity can be controlled by treatment with boiling dilute hydrochloric acid. Floridin XXF is a finely powdered adsorbent, otherwise similar to the more sandy and generally more useful Floridin XS.

Fullers' Earth. Many of the earths used to full cloth are good adsorbents. Like the adsorptive clays they are acid and because of their

relatively low price they are much used in large-scale work for the removal of coloured impurities from fixed oils and many other products. Certain finer varieties of these earths are known as Lloyd's Reagent while some similar products are referred to as Bentonite.

Charcoal. Among the many varieties of activated charcoal are to be found the most powerful adsorbents known. In general, charcoal is not conveniently adaptable to chromatographic work owing to its black colour and the difficulty of eluting substances adsorbed upon it. However, it has occasionally been used with success and has also found application in the modified technique due to Tiselius in which the solvent is forced upwards through the adsorbent column into an optical observation cell arranged immediately above.[7] By maintaining the whole apparatus at constant temperature mixing of the filtrate is prevented and the various fractions of the liquid chromatogram float in layers one above the other. These different layers, if not distinguishable by their colour, can be located by observing variations in their refractive index. In a successful run the filtrate will at first consist of the pure solvent, then a solution of the least strongly adsorbed component of the mixture under examination, followed by solutions of more and yet more strongly adsorbed components. By application of a suitable optical arrangement it is possible to record successive zones as light and dark areas on a photographic plate. While this method overcomes the disadvantage of the black colour of charcoal the difficulty of eluting the adsorbate remains but, nevertheless, Tiselius employed charcoal in many successful experiments directed to the resolution of amino acids and peptides using water as solvent. In the analysis of amino acids it was found that with this adsorbent even a variation of one methylene group caused a large and easily measurable difference in adsorptive property.

Silica Gel. Precipitated silica behaves as a rather weak adsorbent of the acidic type which, so far, has only been used to a limited extent. Silica gel, which ordinarily contains about 20 per cent. of moisture, will adsorb up to 70 per cent. of water without becoming wet in the ordinary sense of the word and Martin and Synge [8, 9] have taken advantage of this fact to evolve their technique of " partition chromatography ". Whereas in normal chromatographic procedures equilibrium is established between a liquid and a solid phase, by using silica gel saturated with water as the adsorbent the phases are both liquid. When substances dissolved in a solvent immiscible with water are passed through the column they become separated according to their differences in partition coefficient, an effect which, in practice is comparable with the differences in adsorptive affinity displayed by the use of ordinary columns. Where the substances to be resolved are colourless it may be possible to produce visible bands by including, in the water used to mix with the silica, indicators or reagents that will react with the components of the sample under test to give coloured products.

Bone Meal. The ordinary commercial product, variously called bone meal, steamed bone flour or feeding bone flour, when completely deprived of fat and sterols by extraction with a mixed solvent consisting of 3 volumes of light-petroleum (b.pt. 40°–60° C.) 1 volume of acetone

and 1 volume of ether was proposed by T. Barton Mann for use in the determination of β-carotene in dried grass and silage.[10] When an extract of the sample, dissolved in light petroleum, is passed through the column chlorophylls and xanthophylls remain firmly adsorbed while β-carotene is readily eluted by running in more light petroleum, whence it can be determined by well established spectroscopic or colorimetric methods. It may well be that this adsorbent will be found useful in various other chromatographic separations.

Sugars. Powdered sucrose is often used but it is important to ensure that the material forming the column is quite dry because its adsorptive capacity is much impaired by the presence of moisture. Other sugars as, for example, lactose, have also proved to be useful in chromatographic work.

Starch. Different samples, even when derived from the same species of plant, vary considerably in adsorptive capacity but none of the conditions governing these differences have yet been determined.

Inulin. This substance, as derived from dahlia tubers, has been employed with considerable success in the resolution of the chlorophylls.

8-Hydroxyquinoline. In powdered form this compound has been proposed for the resolution of mixtures of inorganic ions present in aqueous solution, the chromatograms being developed with dilute acetic acid.

Other Substances Used as Adsorbents. Occasionally the following have been found of value: Calcium Sulphate (hydrated), Sodium Sulphate (anhydrous), Sodium Carbonate (anhydrous), Lead Sulphide, Titania, Asbestos, Kaolin, Magnesium Trisilicate, Magnesium Sesquisilicate and Magnesium Citrate.

Mixed Adsorbents. Apart from the incorporation of acid-washed kieselguhr or other weakly adsorptive powder to accelerate percolations that would otherwise be slow, it is sometimes helpful to employ a mixed adsorbent as, for example, calcium carbonate and alumina. Again, cases may arise where it is expedient to use two or more adsorption layers in which the more powerful adsorbents will be disposed underneath the weaker.

Zeolites. Although not adsorbents in the true sense, the base exchange minerals, the natural and synthetic zeolites, find some application in chromatography as, for example, for the isolation of vitamin B_1 preparatory to its fluorimetric determination. A suitable brand for laboratory work is marketed under the name Decalso.

Synthetic Resins. The capacity of certain synthetic resins to effect ionic exchanges, first reported by B. A. Adams and E. L. Holmes,[11] has led to their use in purification processes. They possess (in addition

to desirable physical properties such as low density, mechanical strength, porosity and flexible texture) a stable chemical structure with a large active internal surface. Two types are available, the one, known under the trade name Amberlite I.R-100, suitable for the adsorption of basic material and the other, Amberlite I.R-4B, which adsorbs acidic substances. The bases of the first will exchange with metallic cations which may then be eluted with dilute hydrochloric acid, while Amberlite I.R-4B adsorbs anions such as chloride ions. Work in this field is still in an exploratory stage but some success has been achieved in the separation of amino acids [12, 13] and J. P. R. Riches has published an interesting report [14] on the use of synthetic resins in the estimation of trace elements.*

Solvents and Eluants

There is no sharp distinction between a solvent and an eluant since there are many cases of the same liquid serving in one instance as a satisfactory solvent from which substances can be adsorbed and in some other circumstances as an eluant for the liberation of adsorbed material. However, in general, non-polar liquids such as light petroleum serve as the best solvents while a polar substance like ethyl alcohol may be taken as typifying a powerful eluant. Mixtures are frequently employed, and it is a common practice to establish the chromatogram by passing a solution of the sample in light petroleum through the column and to use light petroleum containing perhaps 2 or 3 per cent. of methyl alcohol or acetone for the elution. In all work of an experimental character it is most important that pure materials be employed as the presence of even traces of foreign matter may completely alter the character of a solvent so far as its utility for chromatographic work is concerned. In cases where a high degree of precision is important it is sometimes an advantage to employ pure pentane or hexane in place of light petroleum.

Considering a few other solvents, mention may be made of carbon tetrachloride which, while behaving similarly to the higher boiling fractions of light petroleum, has the advantage of being non-inflammable. While carbon disulphide has obvious disadvantages owing to its extremely low auto-ignition temperature and its toxicity, it has nevertheless been quite extensively used for chromatographic work particularly in connection with the resolution of xanthophylls. Where it is found that adsorption from light petroleum is undesirably strong it is frequently advantageous to work with benzene as solvent. Again, mixtures of these solvents often prove useful and by varying the proportions used in the development of the chromatogram it may be possible to regulate the rate at which the bands move down the column.

Ethylene dichloride, methylene dichloride and chloroform are often useful for developing chromatograms as, according to the conditions,

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* These synthetic resins are manufactured by The Resinous Products and Chemical Company, Philadelphia, U.S.A. It does not appear that they are at present available in Great Britain, but arrangements for their distribution are pending.

they occupy a position somewhere between the non-polar solvent light petroleum and polar eluants such as methyl alcohol, ethyl alcohol, acetone, pyridine and organic acids.

Water is sometimes used as a solvent but because the adsorptive force from aqueous solution is generally weak it follows that where adsorption has occurred difficulty may be experienced in finding a satisfactory eluant. However, advantage may often be taken of the fact that the capacity of most adsorbents varies with the hydrogen ion concentration whence by the judicious use of buffers, adsorption, development of the chromatogram and elution may be effected by making suitable alterations in the reaction of the aqueous medium.

Location of Colourless Adsorbed Substances

For the resolution by adsorption of mixtures of colourless substances some method of identification is necessary. Thus in cases where the materials are not too strongly adsorbed the percolate may be collected in portions provided convenient identification tests can be applied. Occasionally, it may be possible to effect separations by passing a solution of the mixture successively through a number of columns each one of which contains a slightly more active adsorbent than that in the preceding column. Sometimes a coloured substance known to be more strongly or less strongly adsorbed may be added to the colourless material which it is desired to isolate whence the coloured band due to the indicator will assist in locating the portion of the column to be treated with eluant.

In certain cases the presence of a colourless adsorbed substance is revealed by examining the column by the light from a quartz mercury vapour lamp which has been screened with a filter of Wood's glass. When exposed in this way to ultraviolet light a number of substances fluoresce but it is important to ensure that any fluorescence which is observed is not due to the adsorbent.

When it is known that the substance to be isolated belongs to a particular group of organic compounds it may be possible to form a coloured derivative before attempting its isolation by adsorption. Thus carbonyl compounds can be combined with 2 : 4-dinitrophenylhydrazine to yield yellow derivatives while azobenzene-*p*-carboxylic acid chloride has been employed to form coloured compounds with sterols and with sugars.

Another device which is sometimes used consists in painting the extruded columns with a reagent that will give a colour with the adsorbed substance. Thus the presence of vitamin A may be revealed by the use of a chloroformic solution of antimony trichloride while a solution of hydrogen sulphide will detect certain metallic ions.

Part II *Applications*

As already indicated, the chromatographic method is essentially valuable for the separation of mixtures of substances, notably in certain cases where other more conventional procedures fail. It follows directly from this fact that many successful applications of the principle are concerned with the concentration and isolation of valuable constituents present in natural products. There are many instances in which chromatography is satisfactorily applied on a large scale, particularly in the relatively simple case of freeing a technical product from undesirable constituents such as naturally occurring pigments. However, when it is required to separate mixtures of several substances into bands there are limitations on the size of column that can be usefully employed. It is obvious that in wide columns, the same quantity of mixture would form much thinner bands than it would in columns of smaller diameter and, in any given case, a limit will be reached beyond which it is impracticable to effect satisfactory division of the several constituents of the mixtures under treatment. This limitation as to the scale of operations will particularly apply when some of the materials to be isolated occur in small quantities relative to the major constituents of the mixture.

With reference to applications to analytical chemistry it is important to emphasise that chromatography is not a principle of analysis in the same sense as gravimetric, volumetric and colorimetric methods. It is never possible to conduct an analysis, or an assay, by the application of chromatography alone and the term " chromatographic analysis " is unfortunate and misleading.

Vitamins

Vitamin A. It was with the help of the chromatographic method that Karrer and his collaborators first isolated an almost pure, although not crystalline, specimen of vitamin A.[15] Halibut-liver oil was saponified at 60° C. with alcoholic potassium hydroxide in an atmosphere of nitrogen and the unsaponifiable portion isolated by shaking out with light petroleum. The solid residue was freed from sterols by dissolving it in hot methyl alcohol, allowing the solution to stand at − 15° C. for several hours and then filtering at the same temperature. The filtrate was stored in the cold for some days to allow of further separation of sterols and, after again filtering, the solution was frozen by means of a mixture of solid carbon dioxide and acetone then, after allowing to warm until just liquid, the vitamin still in solution was separated from a further crop of insoluble material. The filtrate was diluted with water, extracted wth light petroleum, the latter dried and passed through alumina. Vitamin A was adsorbed in the middle of the column, there being a small amount of sterols at the top and substances of lower molecular weight at the bottom. The central zone was eluted with methyl alcohol, the vitamin

transferred to light petroleum and the adsorption repeated twice. The sample of vitamin A thus obtained was sufficiently pure to allow of ultimate analyses being conducted which led to the establishment of the structural formula. The chromatographic method has been quite widely employed in a number of other investigations in connection with this substance but the original isolation of crystalline vitamin A was achieved by means of a freezing technique without recourse to the adsorption principle.

Vitamin D. Chromatography was used by Windaus, Schenck and von Werder[16] to isolate vitamin D_3 from irradiated 7-dehydrocholesterol and also by Brockmann who separated the same anti-rachitically active substance in purified form from tunny-fish liver oil.[17] The procedure adopted in the latter case is especially interesting and is outlined below:—

About 100 g. of a distilled concentrate of the liver oil was dissolved in 2 litres of light petroleum and the solution repeatedly extracted, first with 500-ml. portions of 90 per cent. methyl alcohol, and then with smaller quantities of 95 per cent. methyl alcohol. The combined alcoholic extracts were diluted with water until the alcoholic strength was 50 per cent., the solution then extracted with light petroleum and the latter removed by distillation. The concentration of vitamin D in the residue was 280 i.u. per mg. and 20 g. of this was dissolved in a mixture of 100 ml. of benzene and 400 ml. of light petroleum and the solution passed through a column of " alumina III ". The column was washed with 1200 ml. of the same mixed solvent and the oil remaining after distillation of the filtrate dissolved in 600 ml. of a 1 : 4 mixture of benzene and light petroleum together with 0·1 g. of the dyestuff Sudan III. This solution was passed through a second column of " alumina III " and, using the same solvent, a chromatogram was developed having a bright yellow zone at the top succeeded, first by a pink section then a red and at the bottom a brownish-yellow portion. The red zone comprised the dyestuff and an oil containing 2000 i.u. per mg. of vitamin D. This was dissolved and submitted to the same chromatographic procedure as before when it was found that the red zone contained 0·9 g. of an oil which now contained 5500 i.u. per mg. of the vitamin. Purification was continued by dissolving in light petroleum, shaking the liquid with a 20 per cent. solution of potassium hydroxide in methyl alcohol (80 per cent.), separating the decolorised petroleum layer, evaporating the solvent and dissolving the oily residue in a little hot methyl alcohol. On cooling this solution the bulk of the cholesterol present crystallised out and after this had been separated by filtration the remainder was removed by adding digitonin, evaporating to dryness and extracting the residue with hot light petroleum. After evaporation of the petroleum the residue, which now weighed 0·75 g., was dissolved in a little dry pyridine and a solution of 3 : 5-dinitrobenzoyl chloride in the same solvent added. After warming for a short time, the mixture was allowed to stand for 2 days at 20° C. in an atmosphere of carbon dioxide and was then poured into sodium bicarbonate solution. The aqueous liquid was extracted with benzene and the extract washed successively with sodium bicarbonate solution, dilute acetic acid and water. The benzene was then removed,

the residue dissolved in a 1 : 4 mixture of benzene and light petroleum and the resulting solution chromatographed on " alumina III ". The eluate from the lowest zone gave an oil containing 10,000 i.u. per mg. of vitamin D. This residue was dissolved in hot acetone, methyl alcohol added until a slight turbidity was produced and the mixture allowed to stand until clusters of yellow needle-shaped crystals formed. After washing with light petroleum and recrystallising, this residue was found to melt at 128° to 129° C. and, in fact, consisted of vitamin D_3-dinitrobenzoate. Subsequent hydrolysis with a solution of potassium hydroxide in methyl alcohol yielded a specimen of vitamin D_3 in the form of an oil with a biological activity of 25,000 i.u. per mg.

Vitamin E. Substances possessing anti-sterility activity occur in the unsaponifiable portion of certain fixed oils notably in wheat-germ oil and the effect has been traced to the presence of α-, β- and γ-tocopherol of which the α compound is by far the most potent. The investigation of this group has been facilitated by the use of chromatographic methods, an interesting example being the isolation of α-tocopherol by Karrer and Salomon.[18]

Vitamin K. Application of the chromatographic adsorption methods materially contributed towards the isolation of the two anti-hæmorrhagic vitamins namely K_1, or 2-methyl-3-phytyl-1 : 4-naphthaquinone, and K_2, or 2-methyl-3-difarnesyl-1 : 4-naphthaquinone. Early results were obtained by adsorbing the vitamins on powdered sucrose, washing away pigments with light petroleum, dissolving the sucrose in water and extracting the solution with an immiscible organic solvent.[19] Subsequently, better results were obtained by extracting alfalfa with light petroleum, running the concentrated extract through a column of synthetic zeolite (Decalso), washing through with more light petroleum and then with this solvent containing 10 per cent. of benzene. Fractions taken from the percolate which exhibited vitamin K activity were chromatographed with fresh Decalso and the richest portions of percolate passed through a column of Permutit, fractions being again collected and the one with the highest vitamin concentration poured through fresh Permutit. Final purification was effected by adsorption of the vitamin K_1 on charcoal and subsequent elution with a mixture of ethyl alcohol and benzene.[20] A similar procedure was adopted for the isolation of vitamin K_2 from putrified fish meal.[21] The establishment of the chemical character of these anti-hæmorrhagic factors followed from these and other investigations and the knowledge thus acquired led to the introduction of the simpler synthetic compound menaphthone (2-methyl-1 : 4-naphthaquinone) and its less irritant derivative acetomenaphthone (1 : 4-diacetoxy-2-methylnaphthalene).

Water Soluble Vitamins. In the investigation of this group chromatographic methods have only played a subsidiary rôle. Reference may be made to the work of Cerecedo and Hennessy who successfully isolated vitamin B_1 from rice-polishings by adsorption on artificial zeolite.[22] The adsorbent (Decalso) was washed, first with dilute sulphuric acid at *p*H4, then with hot water. An extract of rice-polishings was partially purified by treatment with barium hydroxide then warmed to 75° C.

and passed through the column. The zeolite was washed with warm water and the adsorbed vitamin B_1 eluted with a hot aqueous solution of ammonium nitrate. The reaction of the eluate was adjusted to *p*H7·5 and the vitamin precipitated as the silver salt.

As would be expected, the separation of flavins by chromatographic treatment of extracts from various plant and animal sources has been quite extensively investigated and in this connection Heilbron, Parry and Phipers[23] isolated riboflavine (lactoflavin) in the form of its acetyl derivative from the fresh-water alga *Cladophora sauteri.*

Pharmaceuticals

Œstrone. A chromatographic method has been used for the purification of this hormone.[24] The acidified urine from pregnant mares was extracted with benzene, the solvent distilled off, the residue dissolved in light petroleum and the hormone in the solution extracted with dilute aqueous alcohol. The resulting alcohol-water solution was extracted with benzene and the latter then passed through a column of slaked lime. Part of the pigment remained adsorbed at the top as a brown zone while a violet band passed slowly downwards. This band and the region immediately above it contained most of the œstrone which was eluted with acetone and crystallised from alcohol. It was also found that the hormone could be recovered by dissolving the œstrone-rich portion of the column in diluted hydrochloric acid and extracting the solution with ether.

In much later work Veitch and Milone have reported the separation by a chromatographic method of the 2 : 4-dinitrophenyl-hydrazones of estrone and equilenin.[25]

Tincture of Cantharides. According to Valentin and Franck[26] cantharidin may be quantitatively isolated from this preparation by the help of a chromatographic separation. A column is charged using a sludge of alumina in acetone and 25 ml. of the tincture drawn through at a rate not exceeding 2 drops per second followed by a wash consisting first of 10 ml. of a 1 : 1 mixture of acetone and chloroform then of chloroform alone. The percolate is collected, the bulk of the solvent removed by distillation and the remainder by a current of air. The residue is dissolved in a mixture of 9·5 ml. of light petroleum and 0·5 ml. of alcohol and the solution allowed to stand for 12 hours. The crystals that deposit are filtered off, washed with the same mixed solvent of petroleum ether and alcohol then dissolved in a little chloroform, the solution transferred to a tared flask, the solvent removed by means of a current of air and the residue of pure cantharidin dried in a desiccator and weighed.

Emodin Purgative Drugs. The appearance of chromatograms given by various members of this series using magnesia as adsorbent have been reported and it is claimed that certain adulterations can be detected.[27]

It is possible that there is scope for more work in this field and a correlation of chromatographic results with biological response might well be of real value.

Alkaloidal Drugs. Based upon the original proposals of Merz and Franck,[28] methods for the assay of preparations of belladonna, stramonium, nux vomica and ergot using chromatographic technique have been evolved by G. W. Brownlee.[29] In all cases a column of alumina about 37 cm. by 1·3 cm. and packed into a tube 60 cm. in length was used and on top was placed a piece of filter paper cut to size and moistened with the menstruum corresponding with that used for making the galenical under examination or, alternatively, a suitably moistened plug of cotton wool was used for preparations of nux vomica and for all soft extracts. For the assay of belladonna and stramonium, a known volume of the galenical (e.g. 10 ml. of liquid extract of stramonium) or, in the case of extracts, a known weight dissolved in a suitable mixture of alcohol and water, is transferred to the top of the column and gentle suction applied. When only 1 to 2 ml. of liquid remains above the adsorbent the sides of the tube are washed with 2 ml. of alcohol (70 per cent.) and the addition of this solvent is continued until the tube is filled. About 35 ml. of percolate is collected and transferred to a separator with the aid of 10 ml. of 0·1 *N* sulphuric acid and 20 ml. of water. The liquid in the separator is washed with two separate 10-ml. portions of chloroform and each chloroform layer is washed with the same 10 ml. of 0·1 *N* sulphuric acid contained in a second separator. The chloroform is rejected, the acid washing transferred to the first separator, the aqueous liquid rendered akaline with ammonia and the alkaloids completely extracted with successive portions of chloroform. The solvent is removed, the residue dried to constant weight at 100° C. then dissolved in 20 ml. of 0·02 *N* sulphuric acid and the excess acid titrated with 0·02 *N* sodium hydroxide using methyl red as indicator.

For preparations of nux vomica the chromatographic part of the procedure is carried out as described above some 40 ml. of percolate being collected which is then transferred to a separator with 10 ml. of 0·1 *N* sulphuric acid and 20 ml. of water. The liquid in the separator is washed with two separate 10-ml. portions of chloroform and each chloroform layer is washed with two separate 10-ml. quantities of 0·1 *N* sulphuric acid. The chloroform is rejected, the three acid liquids mixed, rendered alkaline with ammonia and the total alkaloids completely extracted with successive portions of chloroform. The solvent is removed, the residue dissolved in 5 ml. of alcohol (95 per cent.) the solution evaporated to dryness and the nitration of the brucine and isolation of the strychnine effected by the official method of the Pharmacopœia. Alternatively, the brucine may be eliminated before the preparation is passed through the alumina column. Thus, to 10 ml. of liquid extract of nux vomica is added 5 ml. of 9 per cent. w/v sulphuric acid and 2 ml. of nitric acid (s.g. 1·42). The mixture is allowed to stand for 30 minutes at a temperature of 15° to 20° C. then made slightly alkaline to litmus with 40 per cent. w/v aqueous solution of sodium hydroxide, at once acidified with acetic acid (33 per cent.) and 0·5 to 1 ml. added in excess. This liquid is chromatographed as already described using 70 per cent.

alcohol containing 1 ml. of 33 per cent. acetic acid per 100 ml. as eluant and 40 to 45 ml. of percolate is collected. This is transferred to a separator with 20 ml. of water, rendered alkaline with sodium hydroxide and the strychnine completely extracted with chloroform. The solvent extracts are combined, extracted first with 20 ml. of *N* sulphuric acid then with successive portions of 0·2 *N* sulphuric acid containing 10 ml. of 90 per cent. alcohol per 100 ml. The combined acid layers are washed with two 10-ml. portions of chloroform and each chloroform layer is washed with the same 10 ml. of 0·1 *N* sulphuric acid contained in a second separator. The chloroform is rejected, the mixed acid liquids rendered alkaline with sodium hydroxide and the strychnine again extracted with chloroform. Each solvent layer is washed with the same 10 ml. of water, the chloroform removed by distillation, 5 ml. of alcohol added to the residue, the solution evaporated to dryness, the strychnine dried for 30 minutes at 100° C. then dissolved in 10 ml. of 0·1 *N* sulphuric acid and the solution titrated back with 0·1 *N* sodium hyroxide using methyl red as indicator. As in the official method, the result is multiplied by 1·02 to correct for loss of strychnine in the process.

For the assay of liquid extract of ergot a column is prepared using 25 to 30 g. of alumina and 5 ml. of the sample is run on top. Gentle suction is applied and, when about 1 ml. remains, the sides of the tubes are washed with 2 ml. of a mixture of equal volumes of acetone and water. When only 1 ml. of this remains the elution is continued with acetone (ANALAR grade) and 35 to 40 ml. of percolate collected. This is transferred to a separator, the flask being washed with anæsthetic ether until a total of 120 ml. has been used. The mixture of ether and acetone, containing the alkaloids from the sample, is extracted, first with a 10-ml. portion and then with four successive 5-ml. portions of a 1 per cent. aqueous solution of tartaric acid. The solvents are removed from the mixed acid extracts under reduced pressure and the assay continued as described on page 7 of the Sixth Addendum to the B.P. 1932 commencing with the words " . . . dilute to 50 ml. with water . . . "

In his original paper Brownlee describes the appearance of some of the chromatograms which he obtained when conducting assays of galenicals but the real interest of his work centres on the fact that the alumina columns adsorb the bulk of the extractive material of the drug but allow the alkaloids to pass through with the percolate. However, except in the case of ergot preparations, removal of the pigments is not adequate and it is necessary to include a " shaking out " procedure. Again, the method fails in the case of hyoscyamine where the proportion of vegetable extractive relative to alkaloid is much higher than in either belladonna or stramonium. An interesting proposal for the assay of belladonna has been made by M. Roberts and W. O. James.[30] The method, which was evolved for the determination of alkaloids in plants grown experimentally in connection with genetical studies, is designed for use with small samples. Essentially, the procedure is as follows:—

Approximately 1 g. of the dried leaf powder is evenly wetted with about 6 drops of 0·5 *N* sulphuric acid and excess pigment extracted with 30 ml. of ether. The solvent still remaining with the powder is driven

off by gently warming. The powder is then mixed with approximately 6 drops of dilute ammonia solution (10 per cent. w/w NH_3) and the alkaloids extracted with 60 ml. of benzene in a miniature percolator. With root and stem powders the preliminary ether washing is not necessary. The benzene extract is poured on to a column of alumina 2·5 cm. by 1 cm. arranged immediately above a column of activated silica gel 12 cm. by 1 cm. When the benzene solution has passed through the columns 30 ml. of absolute alcohol is pipetted on to the alumina and when this has run through the short alumina column is removed and 5 ml. of acetone added to the silica. The latter is now deactivated with 4 ml. of ammonia solution (20 per cent. w/w NH_3) and the alkaloids eluted with 50 ml. of chloroform. The solvent from the percolate is removed by evaporation under slightly reduced pressure on a water-bath adjusted to 60° C. The residue is dissolved in a few drops of alcohol, treated with 1 ml. of 0·02 *N* sulphuric acid and back-titrated with 0·01 *N* sodium hydroxide using a Rehberg-type microburette. Before use, the alumina and the silica gel are washed with 50 per cent. acetic acid, then with distilled water and activated by heating for 24 hours in a silica dish at red heat.

The position of the alkaloids and pigments during this procedure is shown in the Table below:—

	Alumina	**Silica**	**Eluted solution**
I. After adsorption of benzene percolate.	*alkaloids* and all pigments except carotenoids.	carotenoids (weakly adsorbed).	carotenoids
II. After elution with alcohol.	mainly decomposition products of chlorophyll.	*alkaloids*	chlorophylls, xanthophylls, etc.
III. After elution with ammonia and chloroform	—	—	*alkaloids*

An extension of this procedure is described by E. M. Trautner and M. Roberts [31] who employ a column of activated silica gel to separate mixtures of hyoscine and hyoscyamine extracted from certain of the *Solanaceae*. These authors found that silica gel, activated as described above, adsorbed both alkaloids strongly from benzene solution but, whereas hyoscine was rapidly eluted by absolute alcohol, hyoscyamine (together with other more basic alkaloids) was moved only slowly and they showed that, if the ratio of hyoscine to hyoscyamine was between

1–4 and 6–1, an almost quantitative separation of the hyoscine could be achieved.

For the assay of *Duboisia myoporoides*, the following details are given:—

Moisten 0·5 g. of the powdered leaf with dilute sulphuric acid, wash with ether to remove the bulk of the pigment and extract the alkaloids with a mixture of equal volumes of chloroform, previously saturated with dry ammonia gas, and ether. Evaporate the extract to dryness, dissolve the residue in chloroform, remove the solvent and again dissolve the residue in chloroform. Determine the approximate proportion of non-volatile bases present by titration with a chloroformic solution of p-toluene sulphonic acid using dimethyl yellow as indicator, calculating the result in terms of hyoscine. Further purify the total alkaloids by transferring to dilute acid and then to ether, remove the solvent from the mixed ether extracts, previously dried, and dissolve the residue in 25 ml. of dry benzene.

Transfer 10 ml. of this solution to a chromatograph column consisting of a 2·5 cm. layer of activated alumina arranged above a column of activated silica gel (12 cm. × 1 cm.) as described by Roberts and James.[30] Elute the combined columns with one portion of 15 ml. of absolute alcohol, followed by two portions, each of 5 ml., of the same solvent. These last extracts are usually free from bases and may often be discarded. Remove the layer of alumina and elute the bases adsorbed on the silica with 40 ml. of ammoniacal chloroform. Dilute the alcohol fraction to 25 ml. and determine its content of tropic esters on a 1 ml. aliquot part by the colorimetric method of Allport and Wilson [32]; from this result calculate the hyoscine content of the drug. Dilute the ammoniacal chloroform eluate to 50 ml., similarly determine the tropic esters colorimetrically, using a 1 ml. aliquot part and determine also the total bases present by evaporating a 25 ml. aliquot part to dryness, dissolving the residue in a little alcohol and titrating with standard acid. From the result of the colorimetric assay on the ammoniacal chloroform extract calculate the hyoscyamine content of the drug. The difference between this figure and that obtained by titration (calculated as hyoscyamine) gives the proportion of alkaloids other than hyoscine and hyoscyamine.

It is pointed out that although the greater part of the hyoscine is eluted from the silica by absolute alcohol, a small fraction is held tenaciously and can only be removed by ammoniacal chloroform; hence the figure for the hyoscine content of the drug tends to be slightly low and that for the hyoscyamine content correspondingly high. The difficulty usually experienced in studying the adsorption of colourless compounds has been overcome by Trautner and Roberts [31] in their work on the separation of hyoscine and hyoscyamine following their observation that the dye dimethylaminoazobenzene (dimethyl yellow) is weakly adsorbed by silica from benzene solution to give a brilliant red colour. If a benzene solution of the bases is now passed through the column, these are preferentially adsorbed and a yellow band of the unadsorbed dye becomes visible. By elution with ether or absolute alcohol, the dye is rapidly

removed; if these solvents are then replaced by benzene or, better, light petroleum containing a little dye, the adsorption zones reappear and it can be seen which have been eluted, separated or spread.

Chlorophylls

Tswett had observed that chlorophylls *a* and *b* (which differ from one another by the fact that in the *b* compound one of the methyl groups of chlorophyll *a* is replaced by an aldehyde group) could be separated chromatographically but he was unable to isolate sufficient of each to permit of subsequent analysis. This task was first achieved by a laborious partition method.[33] It has been shown that chlorophyll undergoes a change when adsorbed on alumina, calcium carbonate or fullers' earth, a fact that must have been realised by Tswett who used sucrose. This adsorbent was also chosen as being the most satisfactory by Winterstein and Stein who, in 1934, published details of this method of separation using chromatographic adsorption technique.[34] These authors dissolved 3 g. of a crude chlorophyll precipitate in 100 ml. of benzene and diluted the solution with 1500 ml. of light petroleum. This solution was passed through a column of powdered sucrose. After all the chlorophyll had been adsorbed, the column was washed, first with a mixed solvent consisting of 14 volumes of light petroleum and 1 volume of benzene and then with pure light petroleum. At this stage three zones were apparent, the lowest blue-green portion contained chlorophyll *a*, the uppermost yellow-green section contained nearly pure chlorophyll *b*, while in the middle of the column a mixture of the two kinds was adsorbed. The pigments were eluted with a mixed solvent consisting of equal volumes of acetone and ether and transferred to the latter by addition of water. The ether was evaporated, the residue dissolved in a small quantity of fresh ether and the chlorophyll precipitated by addition of light petroleum and the solid collected by centifuging. The chlorophyll *b* was further purified by dissolving 0·2 g. in 15 ml. of benzene, diluting to 150 ml. with light petroleum and adsorbing on a column of sucrose. The pigment in the chlorophyll *b* zone was then eluted, purified and isolated by a procedure similar to that described above.

The following alternative procedure for the isolation of the two chlorophylls is of interest as illustrating variations in chromatographic technique.[35] About 0·5 g. of a crude chlorophyll precipitate from alfalfa was dissolved in 75 ml. of ether, 300 ml. of light petroleum added and the resulting solution passed through a column of *neutral* magnesium citrate. The chlorophylls were adsorbed on the upper third of the column, a narrow band of carotene was washed ahead without adsorption and a small zone of pheophytin occurred between this and the chlorophyll. After washing with some 500 ml. of additional solvent the blue-green chlorophyll *a* zone was well defined and contiguous with a light green zone and this in turn with a darker green zone. These three were removed separately with a spatula and the pigments eluted from the adsorbent by solution in acetone. The solution of chlorophyll *a* was transferred to 75 ml. of light petroleum by the addition of water and xanthophyll removed by washing with five 15-ml. portions of 85 per cent. methyl

alcohol. Finally the alcohol was removed by extraction with water, whereupon the chlorophyll *a* precipitated; this was collected and dried, about 0·1 g. in the form of blue-black flakes being obtained. The acetone solution of the pigments from the light green and darker green zones mentioned above was added to a mixed solvent consisting of 4 volumes of light petroleum and 1 volume of ether and the acetone removed by extraction with water. After drying with a little anhydrous sodium sulphate, the solution of pigment was decanted into a column of inulin followed by 300 ml. of the light petroleum-ether mixture. A separate yellow-green chlorophyll *b* zone developed and, after mechanical removal from the column, the pigment was eluted with acetone and about 0·05 g. recovered by the procedure already outlined for chlorophyll *a*.

Carotenoids

It is in the study of this group of compounds that some of the most outstanding achievements of the chromatographic technique have been realised. However, in view of the specialised nature of this work, only a brief allusion to the subject will be included here. As already indicated, it was due to the success which attended the application of chromatography to the separation of certain closely related carotenoids that re-established interest in adsorption methods. The molecules of this group of compounds comprise long aliphatic chains with attached methyl groups. In some of the pigments the ends of the chain are looped into rings like those of the ionones while the observed differences in adsorbability can often be correlated with the number and arrangement of the conjugated double bands. The carotenoids are divided into the following groups:—

1. Carotenes, hydrocarbons mostly of the empirical formula $C_{40}H_{56}$.
2. Xanthophylls, oxy and hydroxy derivatives of the carotenes.
3. Carotenoid Acids, carboxyl derivatives of carotenes.
4. Xanthophyll Esters, esters of the xanthophylls with the fatty acids.

Mixtures comprising pigments severally belonging to all types of carotenoids can be separated into the above groups by a partition technique between immiscible solvents both before and after saponification. Thus, if a solution in light petroleum of material derived from all groups be extracted with 90 per cent. methyl alcohol xanthophylls and carotenoid acids are removed, leaving carotenes and xanthophyll esters in the petroleum. The former pair may be separated by transferring from methyl alcohol to ether and extracting with a weak aqueous solution of alkali which removes the carotenoid acids. The carotenes and xanthophyll esters left in the original light petroleum solution are saponified with alcoholic solution of potassium hydroxide and the xanthophylls thus produced separated from the carotenes by partitioning between 90 per cent. methyl alcohol and light petroleum. By suitable chromatographic manipulations many of the individual members belonging to the

same group can be separated from one another. For details of the methods the original literature should be consulted but outlines of some of the procedures will be found in the books on Chromatography cited in the Foreword.

For the assessment of the nutritional value of dried grass, alfalfa, and certain other vegetable feeding stuffs it is necessary to determine the content of carotenes. Of the three isomerides α-, β- and γ-carotenes only the two first-named can act as precursors of vitamin A. In most leafy materials β-carotene is the preponderating carotenoid present, but it has been shown that grass contains an appreciable proportion of a petrol-soluble compound of this group that does not exhibit the properties of β-carotene and has no biological activity. Having in mind this and other considerations, such as the possibility of β-carotene undergoing spontaneous stereo-isomerisation in solution, the following procedure has been published by W. A. G. Nelson on behalf of the Carotene Committee of the British Crop Driers' Association.[36]

Boil from 1 to 2 g. of grass meal with 50 to 60 ml. of light petroleum (b.pt. 80° to 100° C.) under a reflux for 1 hour. Cool and filter the extract through sintered glass or any other suitable type of filter, or merely decant directly, on to a column of bone meal (2 inches by 1 inch). Rinse the flask and residue with small quantities of light petroleum (b.pt. 40° to 60° C. may be used at this stage), apply suction to the bone meal column, elute with light petroleum (b.pt. 40° to 60° C.) and concentrate if necessary. Estimate the carotene in the eluate colorimetrically.

The bone meal used for carrying out the above procedure should be fat-free material specially prepared for adsorption purposes. The column can be used for a large number of determinations. After repeated use it may be washed with acetone to remove the bulk of the pigments and, after a final wash with light petroleum, the column is ready for further tests. The colorimetric determination is conducted by comparing the colour of the light petroleum eluate with that of 0·025 per cent. aqueous solution of potassium dichromate solution which it has been agreed shall be considered as having the same colour intensity as a light petroleum solution containing 0·158 mg. of β-carotene per 100 ml.

Vegetable and Mineral Oils

It has been observed [37] that if a solution of linseed oil in *n*-hexane be passed through an alumina column the constituents of the oil range themselves in descending order of unsaturation. In the case of fatty acids the adsorption affinities are the reverse of those for the corresponding esters [38, 39] the more saturated acids being most strongly adsorbed from light petroleum. An interesting explanation of this phenomenon, based upon the distribution of electrical charges, has been postulated by K. A. Williams.[40] Apart from the procedures already mentioned in connection with the determination of fat-soluble vitamins, simple chromatographic methods are available for the isolation of certain constituents of fats.

Fat in Mixtures containing Fatty Acids. For the analysis of soap stock fatty acids or acid oils Sylvester, Ainsworth and Hughes suggest dissolving the sample in chloroform or ether, passing the solution through a column of alumina tinted with bromo-thymol blue and washing with the same solvent until the percolate is free from fat.[41] The fatty acids are strongly adsorbed and the separated fat in the percolate can be weighed after removal of the solvent.

Unsaponifiable Matter. The same authors (*loc. cit.*) recommend the following procedure:—

From 2 to 2·5 g. of fat is saponified by treating for 2 hours with boiling 0·5 *N* alcoholic potash. While still slightly warm, the soap solution is transferred to a separator the flask being washed successively with 50 ml. of water and 50 ml. of ether, both these being added to the original liquid. After vigorous shaking and separation, the lower layer is run into the original saponification flask, The upper layer is transferred to another separator containing 20 ml. of approximately 0·1 *N* hydrochloric acid and a drop of methyl orange indicator solution. The soap solution is extracted again in the same way with two further 50-ml. portions of ether, and the combined ether extracts in the second separator are shaken vigorously with the dilute acid. After separation, the aqueous layer, which should still show an acid reaction, is removed and the ether solution is washed with 20 ml. of water. As much as possible of the water is removed, including that in the stop-cock and the stem of the funnel (which should preferably be short) is dried with a strip of filter-paper. The ether extract is then finally dried by the addition of a few grams of anhydrous sodium sulphate. A plug of cotton wool is placed in the top of a column of alumina (tinted with bromo-thymol blue) which is about 10 cm. long by 2 cm. in diameter and the extract of unsaponifiable matter is passed through. The separator and the column are washed with five successive 50-ml. portions of dried ether, special care being taken to wash the end of the stem of the separator, and the lower end of the adsorption tube. The ether is distilled from the percolate and washings in an all-glass apparatus, and the residue of unsaponifiable matter finally heated to constant weight at 80° C.

This chromatographic method of treating the ether extract avoids the laborious series of washings which are otherwise necessary to remove acidic bodies and to effect quantitative isolation of unsaponifiable matter. In the method for the determination of unsaponifiable matter specified by the Society of Public Analysts and Other Analytical Chemists [42] special procedures are recommended for shea butter, rosin, and fats containing metallic soaps but none of these is necessary when the above adsorption method is adopted.

Hydrocarbons in Natural Oils. In 1936, T. Thorbjarnarson and J. C. Drummond [43] demonstrated the presence of the highly unsaturated hydrocarbon squalene in olive oil by passing a solution of the unsaponifiable matter in a mixture of 9 volumes of light petroleum and 1 volume of benzene through a column of activated alumina. That portion of

the unsaponifiable matter which passed through the column they found to consist of hydrocarbons and to have an iodine value of about 345 and from this fraction, after purification, they isolated squalene.

A similar procedure of selective adsorption was followed by Thorbjarnarson, Ruiz and Drummond [44] for the examination of the unsaponifiable matter of marine oils. Fitelson [45] has applied the principle evolved by Thorbjarnarson and Drummond [43] to the determination of olive oil in edible oil mixtures, the amount of squalene in that portion of the unsaponifiable matter derived from the mixture which remains unadsorbed after passing its solution in light petroleum through a column of alumina being determined by halogen absorption using the pyridine sulphate-bromine method of Rosenmund and Kuhnhenn.[46] In the determination of the squalene content of a number of vegetable oils it was found that forty-four samples of olive oil gave values from 136 to 708 mg. per 100 g. with an average of 330 mg., while the content, in mg. per 100 g., of other common edible oils was found to be as follows:— cottonseed, 8; arachis, 28; corn, 28; soya bean, 11; sunflower, 13; teaseed, 12; sesame, 3; and rape, 28. In view of the wide limits for the squalene content of olive oil, it is emphasized that this method is only confirmatory. The same author [47] examined a number of other natural fats for their content of squalene using this principle of selective adsorption and found that the hydrocarbon occurred in a number of vegetable oils as follows:—mustard, 7 mg. per 100 g.; rice bran, 332; grapeseed, 7; almond, 21; coconut, 2; linseed, 4; butter, 7; cod-liver, 31; seal, 35; chicken, 4; lard, 3; beef, 10; and cocoa butter, nil.

The principle of Thorbjarnarson and Drummond may be used in a number of other cases as, for example, in the determination of hydrocarbons present in wool grease and the assessment of the content of mineral oils that may be admixed with fatty oils and waxes generally.[40] For the examination of whale oil extracted on board floating factories using oil fuel as a source of power, E. R. Bolton and K. A. Williams [48] developed the following chromatographic method for the detection and determination of fuel oil in whale and fish oil:—

Filter 20 g. of whale oil at a sufficiently high temperature to maintain all stearine in solution, dissolve in 80 ml. of light petroleum and pass through a column prepared by pouring a suspension of alumina in light petoleum into a tube $\frac{3}{8}$ to 1 inch in diameter and about 16 inches long until, after packing, a column 6 to 8 inches high is produced. When the greater part of the oil solution had passed through the adsorbent, wash with a total of about 500 ml. of light petroleum and continue the suction until the column is free from solvent. Push out the contents of the tube, separate the first inch or so and shake with 50 ml. of ether; filter, remove the solvent by evaporation, dissolve the residue in 50 ml. of light petroleum, and pass the solution through a second column prepared in the same manner as the first. If fuel oil is present in the sample a characteristic black or brown stain is produced on the adsorbent. In the absence of mineral contamination, the stain is yellow. Wash the column as before, push out the alumina, extract the top layers with ether and evaporate the extract. If more than 0·005 per cent. of fuel oil is present in the

original sample, the residue is black or brown; if no contamination exists, it is yellow.

Oxidised Fatty Acids. These acids are characterised by their low solubility in light petroleum and thus differ from the freely-soluble normal acids. The usual method for their determination in vegetable oils consists in the preparation of the free fatty acids by saponification and their extraction with the solvent. When the resulting solution is allowed to stand a precipitate forms which is collected on a filter, washed, dried and weighed. However, the results thus obtained tend to be low due to the solubilising effect of the normal fatty acids present and it has been found [40] that the loss inherent in the procedure can be obviated by passing the light petroleum filtrate through a column prepared from calcium carbonate that has been heated to about 200° C. for 2 hours. Washings from the precipitate are also passed through the adsorbent which is further washed with light petroleum and then eluted with ether; this eluate is evaporated and the residue dried and its weight added to that of the precipitate separated from the original light petroleum solution. Calcium carbonate is used as adsorbent because, in contradistinction to alumina, it does not adsorb fatty acids from light petroleum.

Impurities in Sulphur Olive Oil. This product contains a small percentage of resinous impurities soluble in carbon disulphide and relatively insoluble in light petroleum. It is known that, as for oxidised acids, the extent of their precipitation from light petroleum is influenced by the relative amounts of oil and solvent. Fifty times as much light petroleum as oil is specified for conducting an agreed solubility test but it has been established [40] that the same result can be obtained by dissolving 5 g. of filtered sulphur olive oil in 250 ml. of light petroleum, passing the solution through a column of calcium carbonate, washing with a further 200 ml. of the same solvent, eluting with carbon disulphide, evaporating the eluate and, after drying, weighing the residue.

The Ageing of Turbine Oils. The condition of these oils, which are used continuously over long periods of time, is usually checked by determinations of the neutralisation value, saponification value, demulsification value and viscosity, the approaching end of their useful life being indicated by an abnormal increase in the rate at which these figures rise. An analogy can be drawn between the composition of major constituents of lubricating oils (long-chain hydrocarbons) and of fatty oils (long-chain fatty acids) since ageing is promoted by oxidation at points in the chains and, in both cases, products are formed which are relatively insoluble in light petroleum. K. A. Williams has shown [49] that the oxidation product produced by ageing (which resembles in appearance and physical state the coating on fly-papers and may be termed " dissolved gum ") can be satisfactorily determined by dissolving 5 g. of the sample in 100 ml. of light petroleum, passing the solution through a column of alumina (20 cm. × 2 cm.) and, after washing with an additional 500 ml. of the same solvent, eluting with ether, evaporating the eluate, drying and weighing. Examples are quoted to show that while a specimen of new oil contained only 0·28 per cent. of " dissolved gum " an unusual sample which had been continuously used in a turbine unit for nearly

9 years, largely under adverse war-time conditions, contained 5·35 per cent. while, after further use for another 6 months, a precipitate of gummy sludge became evident, thus endangering the running of the centrifuge and turbine, and the content of " dissolved gum " had risen to 9·29 per cent.

Benzpyrene in Blue Shale Oil. In the distillation of Scottish shale a highly carcinogenic fraction is produced known as blue shale oil. Berenblum and Schoental,[50] using a chromatographic technique, succeeded in isolating 3 : 4 benzpyrene (more usually designated 1 : 2 benzpyrene), a hydrocarbon well known for its powerful action in producing cancer. A sample of the blue fluorescent oil was dissolved in light petroleum, the solution passed through an alumina column, the chromatogram developed with large amounts of light petroleum followed by mixtures of this with increasing proportions of benzene until pure benzene was used. The percolate was collected in fractions and the latter tested for carcinogenic potency against white mice. It was found that the active fractions exhibited fluorescence spectra having the same bands as those given by benzpyrene and the presence of this compound was confirmed by chemical means involving the concentration of the active percolates, oxidation with chromic acid of the material isolated by sublimation in high vacuum, further purification by adsorption from benzene on an alumina column and reductive methylation to produce a mixture of 3 : 6 and 3 : 8 dimethoxy-1 : 2-benzpyrene which was readily identified by the fluorescence spectra. It is of interest to note that a similar analysis applied to an extract of shale did not yield any fractions containing 1 : 2 benzpyrene, thus indicating that this hydrocarbon is formed during the production of the oil by destructive distillation.

Amino-acids

The technique of " partition chromatography ", already referred to on page 11, was first applied to the study of this group of compounds.[8] Thus, mixtures of amino-acids such as are produced by the hydrolysis of proteins were separated by first acetylating the hydrolysate to neutralise the basicity of the amino groups, dissolving in chloroform containing 0·5 to 1·0 per cent. of *n*-butyl alcohol and passing through a column of special silica gel. The latter was prepared by adding 53 per cent. of a saturated aqueous solution of methyl orange to dry silica gel, treating the free running powder with chloroform (saturated with water and containing 1 per cent. of *n*-butyl alcohol) to form a slurry and transferring to an adsorption tube. On development, zones of the different acids form, their positions being revealed by the included indicator turning red. In later work methyl orange, which tended to wash through the column was replaced by pelargonin chloride, the pigment from scarlet dahlia petals [51] and subsequently by the more easily obtainable synthetic indicator, 3 : 6-disulpho-β-naphthalene-azo-N-phenyl-α-naphthylamine.[52] A method of standardising the special silica, analagous to the Brockman and Schodder procedure for alumina (p. 9) has been proposed.[51] A solution of 3 mg. of acetyl-phenyl-alanine and 3 mg. of acetyl-leucine in chloroform containing methyl orange and 1 to 3 per cent. of *n*-butyl

alcohol is poured through a column 1 cm. in diameter and containing 3 g. of the silica gel and the chromatogram developed with the same solvent. When the resulting two acid fractions are separated and titrated the total acid found should be within 2 per cent. of the quantities originally added while recovery of acetyl-phenylalanine in the first zone issuing from the column should be at least 99 per cent. This type of water-saturated silica really constitutes an inert mechanical support for the aqueous phase. Although the optimum conditions for producing and standardising this special form of silica is still matter for debate it has been successfully employed in the amino-acid analysis of such proteins as insulin, edestin, casein and β-lactoglobulin [53] and for the extraction and separation of acids occurring in fruit.[54]

Levi and Terjesen have described a modification of the partition chromatogram in which the silica gel is saturated with a buffer solution instead of with water.[55] By this device the capacity of the column is greatly increased and differences in acid strength as well as differences in partition coefficients determine to what extent substances can be separated. By application of this development it is possible to separate pentenyl penicillin (penicillin I) from benzyl penicillin (penicillin II) both from one another and from other penicillins. Thus, penicillin III (or *p*-hydroxybenzyl penicillin) being a stronger acid than either penicillins I or II, is retained at the top of the column while penicillin K (or heptyl penicillin), being the weakest of them all, tends to be washed down toward the bottom of the column. Following on this work, Goodal and Levi have developed a micro-chromatographic method for the detection and approximate determination of the different penicillins in mixtures.[56]

Returning to the analysis of amino-acids, in 1943 Gordon, Martin and Synge [57] suggested the use of filter paper strips in place of silica gel as the mechanical support for the aqueous phase. For partition chromatography with paper, an organic solvent partially miscible with water, such as phenol, *n*-butyl alcohol or collidine has proved to be the most satisfactory. To run a chromatogram, a strip of Whatman No. 1 filter paper, 1·5 cm. or more in width and 20 to 56 cm. in length, is used. A pencil line is drawn across the strip about 5 cm. from one end, 2 to 4 µl. of solution containing, say, 5 to 15 µg. of each amino acid to be separated, is applied along the centre portion of this line from the tip of a capillary tube and the end of the paper nearest the pencil line is fixed into a specially designed trough containing the solvent saturated with water. The whole is then suspended in a suitable chamber whose atmosphere is saturated with both water vapour and the vapour of the organic solvent used. The temperature in the chamber (which may conveniently consist of a stoneware drain-pipe the top being ground flat and covered by a sheet of glass while the bottom is arranged to stand in a leaden tray containing a supply of the organic solvent and water) should not be allowed to vary appreciably during the period of an experiment. When the solvent has run a convenient distance down the strip (15 to 25 cm. in 6 hr.: 30 to 50 cm. in 24 hr., depending upon the solvent and temperature) the paper is removed and the position of the solvent front is marked. The strip is dried by hanging in an oven at 110° C. then

sprayed with a 0·1 per cent. solution of triketo-hydrindene hydrate (ninhydrin) in *n*-butyl alcohol and dried again. Finally, the paper is heated at 80° C. for 5 minutes and the bands are then outlined in pencil, as fading of the colour takes place after a few days.

The individual amino-acids can be identified by comparison with samples of known constitution having regard to their colour reactions with ninhydrin, varying from blue to orange, and also the ratio of the movement of the band to the movement of the advancing front of the liquid, known as the R_F factor.

In order to achieve distinct separations of amino-acids by means of the one-dimensional paper strip technique it is a necessary condition that the R_F factors differ by more than 10 per cent. With many mixtures this condition will not obtain and to meet the difficulty Consden, Gordon and Martin [58] devised a method of two-dimensional chromatography on paper. In place of the strip a rectangular sheet of filter paper (18 × 22½ inches) is used. The amino-acid solution to be analysed (6 to 12 µl., representing 200 to 400 µg. of protein) is placed near a corner, about 6 cm. from either edge. One edge is dipped in a trough of suitable length containing solvent saturated with water, the sheet of paper being suitably clamped into position by means of a glass support. The whole is enclosed in a suitable chamber prepared in a manner similar to that used for the strip method. After the chromatogram has developed for 24 to 72 hours the square of paper is removed and dried, then turned through a right angle and returned to the trough, the latter, as well as the chamber, having been charged with a second organic solvent saturated with water. The chromatogram is again allowed to develop during 24 to 48 hours and the paper then removed, dried as before, and finally sprayed with ninhydrine solution. The use of two solvents in this way greatly increases the possible resolutions since the R_F value of an amino-acid greatly depends upon the solvent used.

The value of this two-dimensional procedure has been well demonstrated by an investigation into the structure of gramicidin S, an antibiotic extensively used by the Russians in the treatment of war wounds. It had been shown by partition chromatography using a complete hydrolysate with silica gel columns that gramicidin S is a pentapeptide, formed by the linkage of *l*-ornithine, *l*-proline, *l*-valine, *l*-leucine and *d*-phenylalanine, but there was no clue as to the way in which the amino-acids are linked together.[59] The actual arrangement was revealed by fractionating partial hydrolysates, in which the amino-acids were present as di- and tri-peptides, on two-dimensional paper chromatograms.[60] The individual spots corresponding to each di- and tri-peptide were cut out, the material constituting each spot was completely hydrolysed and then re-chromatographed. In this way the di-peptides were identified as α-*l*-valyl-*l*-ornithine, *l*-ornithyl-*l*-leucine, *l*-leucyl-*d*-phenylalanine, *d*-phenylalanyl-*l*-proline. Two other spots were identified as being due to the two tri-peptides, α-valylornithyl-leucine and phenylalanyl-prolylvaline. These results indicate that the five amino acids are linked together in the following sequence: -α-*l*-valyl-*l*-ornithyl-*l*-leucyl-*d*-phenylalanyl-*l*-prolyl- occurring once or repeatedly in an endless peptide chain (cyclopeptide).

Thus it appears that gramicidin S constitutes an entirely new type of anti-bacterial substance. This novel application of chromatographic principles to the structure of proteins has been adjudged as the greatest contribution made in this field since the classical work of Emil Fischer.

Miscellaneous Organic Compounds

Under this heading it is only proposed to include a few random notes since, obviously, the applications of chromatography in this field are almost illimitable. Thus, by way of illustration, the separation of optical isomers has been most interestingly demonstrated by the resolution on alumina columns of the *cis* and *trans* forms of azobenzene and certain of its derivatives.[61, 62] Again, the three isomeric nitro-anilines can be separated by adsorption on lime from solutions in light petroleum.[63] In this instance it was noted that the para compound remained uppermost while the ortho isomer settled as a brownish-yellow band in the lowest part of the column. Similarly, the three isomeric nitro-phenols have been observed to form separate bands in the same order,[64] and it has been shown that the readiness with which these compounds are adsorbed bears a direct relation to their dipoles, the isomer with the greatest dipole forming the upper band.[65]

As might be expected, a mixture of triphenyl-methane and triphenyl-carbinol can be separated by the chromatographic method since the hydrocarbon will pass through the column much faster than the alcohol.[66]

Chromatography has proved to be a most valuable aid in the study of condensed polycyclic compounds and allusion has already been made to its use in isolating 1 : 2-benzpyrene from distilled shale oil. In earlier work, Winterstein, Schön and Vetter [67] isolated impurities such as carbazole, naphthacene and anthraquinone from commercial samples of anthracene, while 1 : 2-benzcarbazole was found in a specimen of chrysene and 2 : 3 : 5 : 6-dibenzo-coumarone was separated from technical pyrene. These same investigators recorded that the intense blue fluorescence of purified anthracene was quenched by the presence of 0·00003 per cent. of naphthacene while Wagner-Jauregg observed that the blue fluorescence of impure acridine was changed to yellow when the compound was purified by adsorption on alumina.[68]

Rotenone present in derris root has been isolated in a state of moderate purity by dissolving the crude resin in benzene and passing the solution through a column of Frankonit KL whence much of the impurity remained on the adsorbent and the method is suggested as a preliminary stage in the routine assay of commercial samples.[69]

In their classic work on the chromatographic separation of sterols, Ladenburg, Fernholz and Wallis [70] first esterified various members of the group by treating with azobenzene-*p*-carboxyl chloride in pyridine

at 100° C. for an hour, then precipitating the coloured esters by the addition of methyl alcohol and recrystallising from benzene and ethyl alcohol. It was shown that certain mixtures of these esters could be separated by adsorption on alumina from benzene solutions, the chromatograms being developed with light petroleum and the separations rendered evident by variations of tint. By this and similar procedures it is possible to separate cholesterol and ergosterol, and stigmasterol from either one or the other of these. The ester of β-sitosterol could not be separated from that of cholesterol and only partially from those of the other two sterols mentioned above. It was concluded that effective separation of sterols by chromatographic means depends essentially upon differences between the number of double bonds in the molecule and not upon their position or the shape of the side chains.

A chromatographic method for the separation and determination of cholesterol and cholesterol esters present in blood has been developed by W. C. Hess [71] in which either serum or plasma is treated with a mixture of equal volumes of acetone and ethyl alcohol, the resulting suspension filtered and an aliquot part of the filtrate evaporated to dryness. The separation is effected by dissolving the residue in light petroleum and passing through an alumina column; both free sterol and sterol esters are adsorbed, the latter being eluted with light petroleum containing 10 per cent. of ether and the free sterols with light petroleum containing 10 per cent. of ethyl alcohol. The esters are saponified and the free cholesterol in each fraction is determined by application of the Liebermann-Burchard method using pure cholesterol as standard.

The *p*-phenylphenacyl esters of fatty acids have a blue fluorescence in ultraviolet light and it has been claimed that these derivatives can be separated from one another by adsorption on silicic acid.[72] It is reported that each member, from acetic to capric, inclusive, could be separated from the one preceding or following it, the higher carbon analogue always appearing at the bottom of the column. The *iso* acids with four, five and six carbon atoms formed a similar series. The *iso*-butyrate derivative could be isolated from the *n*-butyrate, but not the *iso*-valerate from the valerate ester. In contrast with this, methyl ethyl *p*-phenylphenacyl acetate was separated from the *iso*-valerate. The trimethyl acetate was satisfactorily removed from the valerate, the *iso*-valerate, and the *n*-caproate. However, neither the capric and undecylic, nor the lauric and myristic derivatives could be separated.

In concluding this section allusion may appropriately be made to the adoption by S. M. Partridge [73] of the one-dimensional paper strip technique to the separation of reducing sugars and the extension of the principle on a two-dimensional basis by Flood, Hirst and Jones [74] to the quantitative analysis of binary and ternary mixtures of sugars such as galactose-rhamnose and glucose-xylose-arabinose which frequently occur in plant gums. A preliminary report on the separation by partition chromatography using paper supports has also been published by Lugg and Overell.[75]

Inorganic Ions

Chromatographic methods for the separation of inorganic substances are not usually of such practical importance as those evolved for dealing with organic substances because alternative and more effective procedures are available. Starting at the top of the column the more common metallic ions are adsorbed on alumina from aqueous solution in the following order: $As^{\cdot\cdot\cdot}$ $Sb^{\cdot\cdot\cdot}$ $Bi^{\cdot\cdot\cdot}$ [$Cr^{\cdot\cdot\cdot}$ $Fe^{\cdot\cdot\cdot}$ $Hg^{\cdot\cdot}$] $UO_2^{\cdot\cdot}$ $Pb^{\cdot\cdot}$ $Cu^{\cdot\cdot}$ $Ag^{\cdot}$ $Zn^{\cdot\cdot}$ [$Co^{\cdot\cdot}$ $Ni^{\cdot\cdot}$ $Cd^{\cdot\cdot}$ $Fe^{\cdot\cdot}$] $Tl^{\cdot}$ $Mn^{\cdot\cdot}$. The substances enclosed within square brackets are not readily separable by this means. Alumina alters the character of certain adsorbed ions as, for example, those of monovalent mercury which change into a mixture of the metal and mercuric ions. Again, the adsorbent may induce oxidation-reduction reactions between ions in different degrees of oxidation such as cupric and ferrous. If water is replaced by ammonia, both for moistening the column and for washing it, the order of adsorption is altered by reason of the formation of ammine complexes. The change in the relative position of silver occasioned by the formation of the particularly stable ammine complex is especially noteworthy: $Co^{\cdot\cdot}$ $Zn^{\cdot\cdot}$ [$Cd^{\cdot\cdot}$ $Cu^{\cdot\cdot}$] $Ni^{\cdot\cdot}$ $Ag^{\cdot}$. The positions assumed by the complex cations produced by metallic salts in the presence of alkaline solutions of tartrates may also afford possibilities for effecting useful separations. Much of the pioneer work on the application of chromatography to the separation of inorganic substances is due to G. M. Schwab and his collaborators [76-81] while the most interesting result so far obtained in this field has been the separation of binary mixtures reported by Schwab and Jockers.[82] A few months before the appearance of any of the reports just mentioned, E. Lange and K. Nagal published a paper upon the chromatographic separation of the rare earths.[83]

According to Schwab and Dattler [84] many inorganic anions may be resolved upon adsorption columns but the method is limited in value owing to the impossibility of converting many of the acid radicles into coloured products. Starting at the top of an alumina column previously washed with dilute nitric acid some of the common anions are adsorbed in the following order: OH' PO_4''' F' [$Fe(CN)_6''''$ CrO_4''] SO_4'' [$Fe(CN)_6'''$ Cr_2O_7''] Cl' NO_3' MnO_4' ClO_4' S''. As before, the square brackets indicate ions that cannot be readily separated by chromatographic means.

Erlenmeyer and Dahn have suggested the use of 8-hydroxyquinoline as an adsorbent for the separation of certain inorganic ions.[85] It is claimed that this procedure has the advantage that in many cases the ions form coloured bands as, for example, tungstates which exhibit a yellow tint while cupric ions produce a green band. Adsorptions may be made from neutral aqueous solutions while it is often convenient to develop the chromatograms with dilute aqueous acetic acid.

Inorganic substances provide interesting examples of chromatographic " spot " tests. When a piece of filter paper is treated with an adsorbent, generally peptized alumina, and a developer (such as, for

example, 8-hydroxyquinoline) it acts like a chromatographic column towards a solution containing constituents suitable to the developer and exhibits coloured zones specific to the constituent, solvent, developer and *p*H. For a binary mixture, say iron and molybdenum, Flood [86] has suggested a formula connecting the radius of the zones with the concentration of the constituents. In later work, P. P. Hopf found that Flood's formula holds in most cases but recommended limiting the method to comparative analyses.[87] Thus, if three drops of a solution containing constituents A and B are placed on a suitable paper at regular intervals during the reaction and two ring-like zones are produced, the first one (for A) being bounded by radii r_1 and r_2 and the second one (for B) by radii r_3 and r_4, and if the initial concentrations of A and B are a and b respectively, then

$$a = \pi (r_1^2 - r_2^2) K_a$$
$$\text{and } b = \pi (r_3^2 - r_4^2) K_b$$

and the values of the constants K_a and K_b can be calculated. It follows, therefore, that

$$\frac{a}{b} = \frac{(r_1^2 - r_2^2) K_{a/b}}{(r_3^2 - r_4^2)}$$

where $K_{a/b} = K_a/K_b$.

The paper used for this work should be tested by submitting 5 g. to wet oxidation in the usual way with a mixture of sulphuric and nitric acids, diluting the cooled residual sulphuric acid to about 100 ml. with water and, after filtering, rendering slightly alkaline with ammonia and adding a little solution of 8-hydroxyquinoline in dilute acetic acid when no colour change or precipitate should be evident. A paper passing this test should be dipped into water in such a way that 1 inch is submerged and 3 inches remains clear: if the paper is suitable, the water should be drawn up to 2 inches above the surface by capillary forces within 1 minute.

The paper is prepared by dipping in a peptized alumina solution or into fairly concentrated hot starch solution. Alternatively, it is dipped in sodium aluminate solution, dried, and then dipped in sodium carbonate solution. In each case it is washed for 5 hours at 20° to 25° C. with stirring, soaked in the chosen developer, washed by immersion in water and air-dried at room temperature. The peptized alumina is prepared [88] by precipitating a hot aqueous solution of aluminium chloride with a slight excess of ammonia and thoroughly washing the hydrated oxide. A considerable excess of this oxide is treated with a hot 8 per cent. aqueous solution of acetic acid by mixing and frequently stirring during 1 hour. The mixture is then centrifuged for 15 minutes to remove the undissolved alumina. The resulting sol is cloudy and viscous but fairly stable.

In illustration, Hopf cites, among others, the following examples:

Ferromolybdenum. A 2g.-sample of the powdered metal was warmed with nitric acid until no more brown fumes appeared, 10 ml.

of 10 *N* hydrochloric acid added, the mixture evaporated to low bulk, then 15 ml. of 18 *N* sulphuric acid added and the liquid heated to fuming. After allowing to cool, 50 ml. of water was added, the preparation boiled, filtered, ammonia added until a turbidity was produced and this was just re-dissolved by the addition of sulphuric acid. When this solution was spotted on to a paper impregnated with peptized alumina and 8-hydroxyquinoline an outer black zone (Fe), separated from an inner yellow spot (Mo) by a blank zone was produced. For this solution and paper, the factor $K_{Fe/Mo}$ had been found to be 0·81. A sample known to contain 73·2 per cent. of molybdenum gave a factor Fe/Mo = 0·346 (actual: 0·35). In this case, since the molybdenum spot remains at the centre, $r_4 = 0$.

Ferromanganese. A test solution was made to contain about 3 per cent. of metal and neutralised with ammonia to incipient precipitation and two drops spotted on to paper prepared with peptised alumina and formaldoxime hydrochloride and, after drying, exposed to ammonia vapour. A standard stain with a metal of Fe/Mn = 0·01081 was found to give $r_1 = 39·17$ mm., $r_2 = 34·00$ mm., $r_3 = 28·8$ mm. and $r_4 = 0$. From this, $(r_1^2 - r_2^2)/(r_3^2) = 0·456$, *i.e.* $K_{Fe/Mn} = 0·0237$. A stain produced by a metal of unknown composition gave the radial ratio as 0·363, *i.e.* Fe/Mn = 0·363 × 0·0237 = 0·0086. The analytical result showed the sample to have the ratio Fe/Mn = 1 : 112·5 = 0·00889.

Alkali Metals. An aqueous solution containing 5 per cent. of sodium acetate, 5 per cent. of ammonium chloride and 5 per cent. of potassium chloride, to which 2 per cent. of acetic acid had been added was spotted on to paper prepared by dipping into hot starch solution, drying, immersing in a developing agent consisting of an aqueous solution of violuric acid (*iso*-nitroso-barbituric acid) and again drying.[89] A spot with an inner blank zone was followed by a red sodium zone ($r_6 = 0·95$ cm., $r_5 = 1·50$ cm.), a pink potassium zone ($r_4 = r_5$, $r_3 = 1·90$ cm.), a blank zone and a blue ammonia zone ($r_2 = 2·45$ cm., $r_1 = 2·475$ cm.). From these the following areas were calculated: Na = 4·24 cm.², K = 4·27 cm.², $NH_4 = 4·71$ cm.². Using a solution containing half the concentrations of potassium chloride and ammonium chloride, the amount of sodium chloride being as before, the radii became $r_6 = 1·25$, $r_5 = 1·70$, $r_4 = r_5$, $r_3 = 1·890$, $r_2 = 2·14$ and $r_1 = 2·315$ cm. whence the following areas were calculated: Na = 4·24 cm.², K = 2·15 cm.², $NH_4 = 2·40$ cm.².

Conclusion

The foregoing outline account of some of the applications of chromatography should be sufficient to convince the reader of the utility and future possibilities of the principle. As an aid in the study of complex organic mixtures, particularly those of natural origin it is undoubtedly of outstanding value while it is no less useful as a means of isolating substances occurring in small concentration in commercial products, either as essential constituents or as impurities.

Hitherto, no allusion has been made in this monograph to the theory of chromatographic separations since, in actual practice the approach has been essentially empirical. However, those interested in the theoretical aspects of the spatial separation of substances on columns of adsorbents are recommended to study the mathematical treatment of the subject as enunciated by J. N. Wilson [90] and also the theory advanced by Martin and Synge to account for the phenomenon of partition chromatography.[8] Further work on the theoretical side has been published by De Vault [91] and also by Weiss[92] and these investigators have shown that certain assumptions made by Wilson are not justified. In another approach, Weil-Malherbe, using benzpyrene under different conditions, measured the volumes of solvent required to develop the column until the front edge of the zone reached the bottom and concluded that, where the only variable in a chromatographic system is the nature of the solvent, the volume required is a measure of eluant power.[93]

In the foregoing pages a brief indication has been given of the advances already made in the theory of the relation between adsorption phenomena and the chemical nature of the adsorbed substance but much more extended accounts are to be found in the books on chromatography mentioned in the Foreword.

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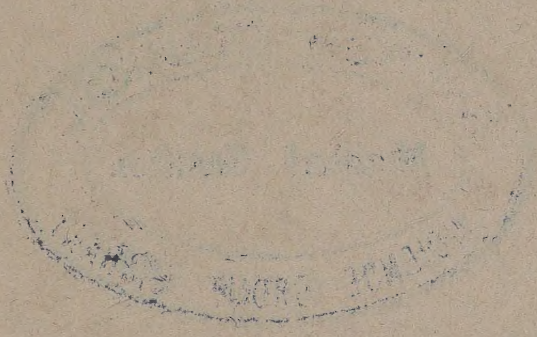

B.D.H.